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Carotenoid–membrane interactions in liposomes: effect of dipolar, monopolar, and nonpolar carotenoids*

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Electron paramagnetic resonance (EPR) spin-labeling methods were used to study the effects of carotenoids on the physical properties of saturated phosphatidylcholine (PC) membranes to evaluate the contribution of the terminal hydroxyl groups of xanthophyll molecules to the carotenoid-membrane interaction. Effects of the dipolar, terminally dihydroxylated carotenoid lutein on membrane phase transition, fluidity, order, and polarity were compared with those of monopolar (β-cryptoxanthin) and nonpolar (β-carotene) carotenoids. These effects were monitored at the membrane center as a function of the amount of the carotenoid added to the sample and as a function of temperature for fluid-phase membranes. PC membranes with different thickness (from 12 to 22 carbons in alkyl chains) were used. Carotenoids shifted to lower temperatures and broadened the main phase transition of PC membranes. They decreased the membrane fluidity and increased the order of alkyl chains. Carotenoids also increased the hydrophobicity of the membrane interior. These effects were the strongest for lutein, significantly weaker for β-cryptoxanthin, and negligible for β-carotene. They decreased with the increase of the membrane thickness. Presented results suggest that anchoring of carotenoid molecules at the opposite membrane surfaces by polar hydroxyl groups is significant in enhancing their effects on membrane properties. This manuscript also shows the ability of EPR spin-labeling methods to monitor different membrane properties that can be applied in biotechnological studies with the use of liposomes.

Keywords: macular xanthophylls, lutein, β-cryptoxanthin, β-carotene, lipid bilayer, EPR, spin labeling, AMD

INTRODUCTION

There is evidence supporting a protective role of lutein and zeaxanthin in delaying age-related vision loss due to age-related macular degeneration (AMD) and cataract formation (Mares, 2004; Cho *et al*., 2004). Only lutein and zeaxanthin (see Fig. 1 for their structures) are selectively accumulated in membranes of the eye retina from blood plasma (where more than 20 other carotenoids are available (Khachik *et al*., 1997)) at an extremely high concentration (Landrum *et al*., 1999). Another carotenoid, *meso*-zeaxanthin, is presumably formed from lutein in the retina (Landrum & Bone, 2001). There are several mechanisms that should be considered in understanding why lutein and zeaxanthin may be protective to the eye. High-energy short-wavelength blue light promotes the formation of reactive oxygen species that can initiate lipid peroxidation in membranes of the human retina, which is a well-oxygenated tissue rich in long-chain polyunsaturated fatty acids. Absorption of this light prior to the formation of reactive oxygen species can be considered an indirect antioxidant action. The most accepted mechanism is through a direct antioxidant action of lutein and zeaxanthin that includes quenching of reactive oxygen species once they are formed. Localization of macular carotenoids in the lipid-bilayer portion

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Abbreviations: AMD, age-related macular degeneration; DSC, differential scanning calorimetry; DXPC (where X = L, M, P, S, B), dilauroyl, dimyristoyl, dipalmitoyl, distearoyl, dibehenoyl, respectively; EPR, electron paramagnetic resonance; PC, phosphatidylcholine; SASL, stearic acid spin label.

of membranes of the human retina is perfect for these actions. However, an involvement of specific xanthophyll-binding proteins in the uptake, stabilization, metabolism, and antioxidant functions cannot be ruled out. Recently, the Bernstein's group (Bhosale *et al*., 2004) identified and characterized a membrane-associated xanthophyll-binding protein that binds zeaxanthin, but not lutein, with high specificity and affinity.

In his review, Krinsky (2002) discusses selective accumulation of lutein and zeaxanthin in the primate retina. He mentions that the ability to filter out blue light could explain the presence of colored carotenoids in the macular region, but does not explain why only two xanthophylls, lutein and zeaxanthin, have been selected from blood plasma. Also, the ability of lutein and zeaxanthin to quench singlet oxygen (Cantrell *et al*., 2003) and to scavenge free radicals (Stahl *et al*., 1998) is not better than that of other carotenoids. Therefore, it must be some specific property of these xanthophylls that could help explain their selective presence in the primate retina. One such property is their disposition in biological membranes. In the human retina, lutein and zeaxanthin are accumulated in the Henle's fiber layer composed of photoreceptor axons (Bone & Landrum, 1984; Bone *et al*., 1992). It was also shown that these carotenoids are present in the rod photoreceptor outer segments (Sommerburg *et al*., 1999; Rapp *et al*., 2000). Their content in photoreceptor outer segment membranes is about ten times lower than that in Henle's fiber layer (Rapp *et al*., 2000). The total carotenoid concentration shows a peak in the central fovea reaching a value about 100 times that of the peripheral retina. Additionally, there is a different ratio of lutein to zeaxanthin comparing the central fovea and the more peripheral regions of retina, with zeaxanthin predominant in the central fovea, and lutein in the periphery (Bone *et al*., 1988; Handelman *et al*., 1988). It is unknown yet what causes this different distribution of lutein and zeaxanthin through the retina.

Macular carotenoids have poor aqueous solubility, and therefore in the human macula they are present in the lipid-bilayer membranes or associated with proteins. Here, we investigated the interactions of carotenoids with lipid-bilayer membranes, paying special attention to the contribution of hydroxyl groups at the ends of xanthophyll molecules to this interaction. For this reason we compared the effects of dipolar carotenoids (lutein and zeaxanthin) on membrane properties with those of monopolar (β-cryptoxanthin) and nonpolar (β-carotene) carotenoids (see Fig. 1 for their structures).

Another goal of this work is to show how the conventional EPR spin-labeling methods and the application of liposomes as a model of biological membranes can be useful in the investigation of different membrane properties. Using one spectroscopic technique but different approaches it is possible to get a vast amount of information even from one membrane location, in this case the membrane center (C16 position). The obtained information includes: lipid alkyl chain order and rotational motion, lipid phase transition, and hydrophobicity of the membrane interior.

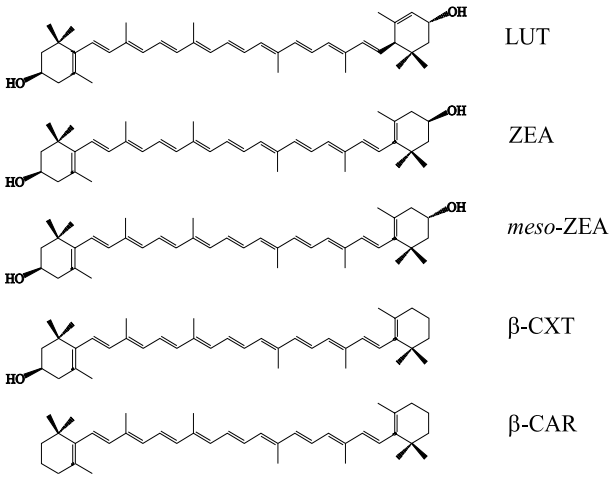

Figure 1. Chemical structures of carotenoids used in this work.
Structures of dipolar, terminally dihydroxylated carotenoids, lutein (LUT), zeaxanthin (ZEA), and *meso*-zeaxanthin (*meso*-ZEA), as well as monopolar (β-cryptoxanthin, β-CXT) and nonpolar (β-carotene, β-CAR) ones are included.

MATERIALS AND METHODS

Materials. All phosphatidylcholines (PCs) were purchased from Sigma (St. Louis, MO, USA) and stearic acid spin labels (SASLs) from Molecular Probes (Eugene, OR, USA). All experiments were performed with 16-SASL, providing information about the membrane center. Only the hydrophobicity profiles presented in Fig. 9 were obtained using seven lipid spin labels with the nitroxide moiety located at different depths in the membrane. Lutein was purchased from Kemin Industries (Des Moines, IA, USA) and β-carotene (*trans*) came from Sigma. Zeaxanthin and the monopolar carotenoid β-cryptoxanthin were a gift from Hoffmann-La Roche (Basel, Switzerland). Chemical structures of lipids, 16-SASL, and lutein and their localization in lipid bilayer membranes are presented in Fig. 2.

Preparation of liposomes. The membranes used in this work were multilamellar dispersions of the PC investigated containing 1 mol% of spin label and 0, 1, 3, or 10 mol% carotenoid added to the sample during preparation. Briefly, these membranes were prepared by the following method (Wisniews-

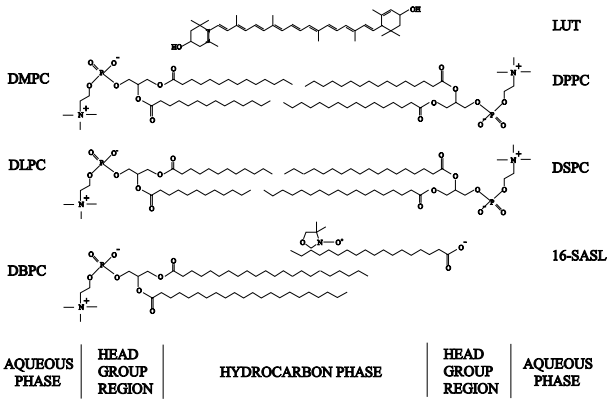

Figure 2. Schematic drawing of the PC-carotenoid bilayer.
Scheme includes structures of lutein, 16-SASL, as well as DLPC, DMPC, DPPC, DSPC, and DBPC. Approximate localizations across the membrane are also illustrated. However, since alkyl chains tend to have many *gauche* conformations, the chain projected to the membrane normal would be shorter than that depicted here and the hydroxyl groups of the rigid carotenoid molecule would be placed closer to the membrane surface in liquid-crystalline phase membranes.

ka & Subczynski, 1998). Chloroform solutions of lipids, carotenoids, and spin labels (containing 5 μmol of lipid) were mixed to attain desired concentrations of the compounds, chloroform was then evaporated with a stream of nitrogen, and the lipid film on the bottom of the test tube was thoroughly dried under reduced pressure (about 0.1 mm Hg) for 12 h. A buffer solution (usually 0.5 mL) was added to the dried film at a temperature above the phase-transition temperature of the PC membrane and vortexed vigorously. The buffer (0.1 M boric acid, pH 9.5) was used to ensure that all SASL probe carboxyl groups were ionized in the membranes (Kusumi *et al*., 1982). The lipid dispersion was centrifuged briefly at 16000 × *g* for 15 min at 4°C and the loose pellet of multilamellar liposomes (about 20% lipid w/w) was used for EPR measurements. All preparations and measurements with carotenoids were performed in darkness or dim light and, when possible, under nitrogen or argon. It should be mentioned here that the carotenoid content indicated in this work as mol% is equal to the amount of carotenoid added to the chloroform solution of lipids before preparation of the liposomes (see also the Discussion section).

Conventional EPR and phase transition. For EPR measurements the sample was placed in a 0.9 mm i.d. gas-permeable capillary made of the methylpentene polymer TPX (Hyde & Subczynski, 1989). The capillary was placed inside an EPR Dewar insert and equilibrated with nitrogen gas, which was also used for temperature control. The sample was thoroughly deoxygenated to obtain the correct line shape. This is especially important near the phase-transition temperature, because the oxygen concentration-diffusion product changes abruptly at the phase transition of the membrane (Subczynski *et al*., 1989). EPR spectra were obtained with an X-band Varian E-109. For phase-transition measurements, the temperature was regulated by passing the nitrogen gas through a coil placed in a water bath, and was monitored using a copper-constantan thermocouple that was placed in the sample just above the active volume of the cavity (Wisniewska *et al*., 1996). Temperature was regulated with an accuracy better than 0.1°C. The temperature was always lowered by adding a small amount of cold water to the water bath with rapid agitation, permitting a very low rate of temperature change of 2°C/h. To avoid heating-cooling cycles, a temperature-controlling unit was not used. For measurements below room temperature, special care was taken not to raise the temperature during cooling. In the case of measurements with DLPC membranes, undergoing the phase transition at –1°C, a propanol bath was used to which small portions of liquid nitrogen were added. It was shown (Wisniewska *et al*., 1996) that when 16-SASL was used, the EPR experiments provided results similar to differential scanning calorimetry (DSC) measurements. In all measurements of the phase transition, the concentration of 16-SASL was 1 mol%.

To measure the hydrophobicity of the membrane interior, the hyperfine interactions of nitroxide that depend on the local polarity around the free radical fragment (Grifith *et al*., 1974) were used. In one approach, the isotropic hyperfine coupling constant, a_0, of 16-SASL was measured for fluid-phase membranes as shown in Fig. 3B. This constant re-

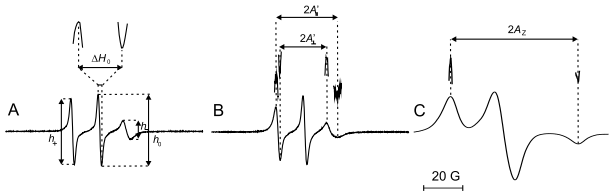

Figure 3. EPR spectra of 16-SASL in DMPC membranes in the absence (A) and presence of 10 mol% lutein at 25°C (B) and at –165°C (C).
Measured values for evaluating rotational correlation times and order parameters are indicated. The rotational correlation time was calculated according to Berliner (1978) from the linear term of the linewidth parameter (τ_{2B} is in second if ΔH_0 is in Gauss). $\tau_{2B} = 6.51\times10^{-10}\times\Delta H_0\left(\sqrt{\frac{h_0}{h_-}}-\sqrt{\frac{h_0}{h_+}}\right)$ The order parameter S was calculated according to Marsh (1981) using the equation $S = 0.5407\ (A'_{II}-A'_{\perp})/a_0$, where $a_0 = (A'_{II}+2A'_{\perp})/3$. In (C) the measured $2A_Z$ value (z-component of the hyperfine interaction tensor) is indicated. The outer wings were also magnified by recording at 10-times higher receiver gain. The peak-to-peak central line width was recorded with expended abscissa (magnetic field scan range by a factor of 10).

flects the polarity (or hydrophobicity) of the membrane center.

In another approach, the *z*-component of the hyperfine interaction tensor, $A_{Z,}$ was determined directly from the EPR spectra of the spin label for samples frozen at –165ºC, as shown in Fig. 3C. Both methods of determining the membrane hydrophobicity have advantages and disadvantages, which we discuss in the section: Hydrophobicity of membrane interior.

RESULTS

Phase transition

The main phase transition of dilauroyl PC (DLPC, containing 12 carbon atoms in alkyl chains (C12)), dimyristoyl PC (DMPC, C14), dipalmitoyl PC (DPPC, C16), distearoyl PC (DSPC, C18), and dibehenoyl PC (DBPC, C22) membranes was monitored by observing the amplitude of the central line of the EPR spectra of 16-SASL (h_0 in Fig. 3A). The phase-transition temperature, T_m, and the width of transition, $\Delta T_{1/2}$, were operationally defined from the EPR data as shown in Fig. 4 (Wisniewska *et al*., 1996).

The influence of carotenoids on the transition temperature and on the sharpness of the transition is the strongest for lutein, significantly weaker for β-cryptoxanthin, and negligible for β-carotene. These effects are summarized in Fig. 5 where shifts of T_m induced by addition of 10 mol% carotenoid are plotted as a function of the membrane thickness. It can be seen that the effect of lutein and β-cryptoxanthin is the strongest for DLPC and decreases with the increase of the membrane thickness. In thin membranes both carotenoids decrease the T_m, which is probably due to the location of the ionone rings of the xanthophyll molecules between polar head groups. This localization causes separation of these groups and an increase of water penetration into this region (Wisniewska & Subczynski, 1998). For DBPC (the thickest membrane investigated here) carotenoids even increase the phase-transition temperature. It should be pointed out that in thick membranes (DBPC with a thickness of the glycerohydrocarbon region of 33.3 Å), the xanthophyll molecule with a length of 30.2 Å is located deeply in the bilayer (Subczynski *et al*., 1993). The contact between lipid head gropus is restored (they "close" above the carotenoid molecule), decreasing water penetration into this region (Wisniewska & Subczynski, 1998) and decreasing the effect on the phase-transition temperature. It is known that the distraction of the organization of polar groups in the lipid bilayer is a significant cause of the decrease of the phase-transition temperature while the ordering effect on lipid hydrocarbon chains should increase this temperature (Gennis, 1989). In thick membranes the ordering effect of xanthophylls "wins" and, as a result, the phase-transition temperature increases. The effect of β-carotene is negligible for all of these membranes. Our EPR measurements are in principal agreement with DSC measurements (Kolev & Kafalieva, 1986; Kostecka-Gugala *et al*., 2003). We should point out here that the EPR measurements were made from cooling experiments (to make sure that the specimen observed was in thermal equilibrium) whereas the DSC measurements were made mainly from heat-

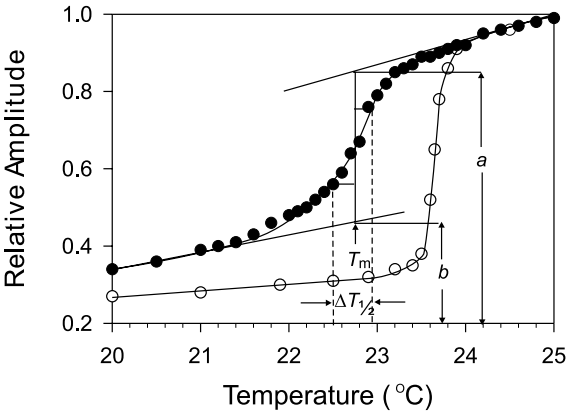

Figure 4. Normalized amplitude of the central peak of the EPR spectra of 16-SASL plotted as a function of temperature (cooling experiments) in DMPC bilayer. Experiments were performed in the absence (○) and presence of 3 mol% lutein (●). Definitions of T_m and $\Delta T_{1/2}$ are shown. T_m is the midpoint temperature at which the normalized EPR signal amplitude equals $(a + b)/2$, where a and b are, respectively, intensities at the given temperatures in the extended linear portions of the upper and lower ends of the transition curve. As the sharpness of the transition, we employed the width $\Delta T_{1/2}$, which is defined by two temperatures at which the EPR signal amplitude is $(a + 3b)/4$ and $(3a + b)/4$.

Table 1. $\Delta T_{1/2}$ values for PC membranes in samples containing 0 or 10 mol% carotenoids.

Carotenoid added	$\Delta T_{1/2}$ (ºC)				
	DLPC	DMPC	DPPC	DSPC	DBPC
No	0.6	0.15	0.3	0.6	0.4
LUT	1.2	2.3	1.9	1.7	0.4
β-CXT	1.2	1.4	1.3	1.25	n.e.*
β-CAR	0.6	0.35	0.5	0.85	0.4

*n.e. – not evaluated

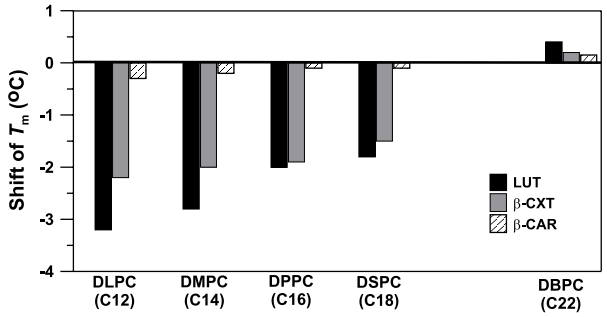

Figure 5. Shifts of the main phase-transition temperature, T_m, of PC membranes induced by the addition of 10 mol% carotenoid to the sample.
Negative values indicate a decrease of T_m. Notice that the x axis indicates the investigated lipid as well as the number of carbon atoms in its alkyl chains. For values of $\Delta T_{1/2}$ see Table 1.

ing experiments, and the rate of temperature change was far smaller in the EPR observations than in the DSC measurements.

The effect of carotenoids on the cooperativity of phase transition (on $\Delta T_{1/2}$) is stronger for DMPC and DPPC membranes as compared with the negligible effect for DBPC membranes (Table 1). This effect increases with the amount of carotenoids added to the sample. The effects on T_m are the strongest for lutein and negligible for β-carotene. However, the difference between lutein and β-cryptoxanthin decreases for thicker membranes (DPPC and DSPC) as compared with membranes made from DLPC and DMPC.

Fluidity of membrane interior

The rotational correlation time of 16-SASL for all investigated PC membranes prepared without and with the presence of 1, 3, or 10 mol% carotenoids was calculated from EPR spectral parameters as shown in Fig. 3A. The rotational correlation time was used as a measure of the membrane fluidity. The addition of β-carotene to the sample up to 10 mol% does not affect the rotational motion of 16-SASL in all investigated membranes and at all temperatures. The addition of lutein significantly increases the rotational correlation time with some sign of saturation at 10 mol%. The effect of β-cryptoxanthin is smaller than that of lutein for DLPC membranes, showing clear saturation at about 3 mol%, but becoming practically the same for thick DSPC and DBPC membranes. Figure 6 summarizes the effects of carotenoids on the rotational motion of the nitroxide moiety of 16-SASL measured for temperatures well above the phase-transition temperature of the host membrane. It is clearly seen that the effect of lutein is strong for all membranes. It is also clear that the effect of β-cryptoxanthin on the rotational motion of 16-SASL depends on membrane thickness.

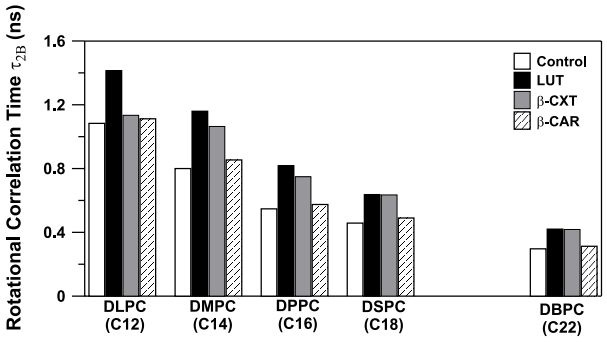

Figure 6. Changes in the rotational correlation time, τ_{2B}, of 16-SASL in PC membranes induced by the addition of 10 mol% carotenoid to the sample.
Results were obtained well above the main phase-transition temperature of PC membranes (20°C for DLPC, 35°C for DMPC, 50°C for DPPC, 60°C for DSPC, and 75°C for DBPC) and plotted as a function of the number of carbon atoms in PC alkyl chains.

Order of membrane interior

Figure 7 summarizes the effects of 10 mol% carotenoids added to the sample on the order parameter of 16-SASL in all investigated PC membranes measured for temperatures well above the phase-transition temperature of the host membrane. The order parameter was calculated from parameters measured directly from the EPR spectra as shown in Fig. 3B. Similar to the case of the rotational motion, the effect of β-carotene on the order parameter is negligible independently of the thickness of the PC bilayer. The effect of lutein is greater than that of β-cryptoxanthin for thin PC membranes and similar for thick PC membranes. The shorter the length of the PC alkyl chains, the greater the ordering effect of lutein and β-cryptoxanthin.

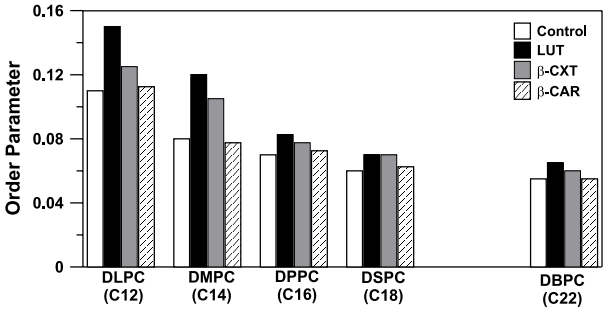

Figure 7. Changes of the molecular order parameter of 16-SASL in PC membranes induced by the addition of 10 mol% carotenoid to the sample.
Results were obtained well above the main phase-transition temperature of PC membranes (see Fig. 6 for temperatures) and plotted as a function of the number of carbon atoms in PC alkyl chains. Because of the sharpness of the EPR lines for 16-SASL and the method of measurements of A'_{II} and $A'_{\perp}$ (see Fig. 3B), the accuracy of measurements of S is ± 0.0025.

Hydrophobicity of membrane interior

We used the isotropic hyperfine coupling constant, a_0, of 16-SASL as a measure of the hydrophobicity of the membrane interior in different PC membranes for samples containing 0 or 10 mol% lutein, β-cryptoxanthin, or β-carotene (see data presented in Fig. 8). A decrease of the a_0 value indicates an increase of hydrophobicity at the 16-SASL position. It can be seen that the addition of lutein causes a significant increase in hydrophobicity in the DLPC and DMPC bilayer while the effect of β-cryptoxanthin is considerably weaker. There is no difference between the effects of lutein and β-cryptoxanthin in DPPC, DSPC and DBPC membranes, although their effects in these membranes are rather weak. In all PC membranes the addition of β-carotene to the sample does not affect the membrane hydrophobicity.

We think that the obtained data correctly reflect the relative changes of the hydrophobicity and give useful information needed for comparison of the effects of dipolar, monopolar, and nonpolar carotenoids on membrane properties. We should, however, warn readers that for the method of a_0 estimation, EPR measurements have to be performed at temperatures well above the phase-transition temperature (Marsh, 1981; 1985). Therefore, for thick membranes like DSPC and DBPC, the measurements of a_0 have to be performed at temperatures as high as 70°C and 80°C, respectively. At these temperatures, the vertical fluctuations of the nitroxide group of 16-SASL towards the membrane surface are significantly increased (Yin *et al*., 1990; Yin & Subczynski, 1996) providing an averaged a_0 value from different depths within the membrane. We think this is the reason why the observed changes of hydrophobicity in thick membranes are negligible. Therefore, we also present data obtained from frozen liposome suspensions. In this approach the local hydrophobicity is monitored by observing the *z*-component of the hyperfine interaction tensor (A_Z) of the nitroxide spin label in a frozen suspension of the membrane at –165°C (Grifith *et al*., 1974). At this temperature, the molecular motion is not detected and only solvent effects on the spectrum are measured. With an increase of the solvent polarity, A_Z increases. The magnetic parameters (including hyperfine interaction tensors) of spin labels in the membrane determined at lower temperatures have been shown to correlate well with those at physiological temperatures (Kusumi & Pasenkiewicz-Gierula, 1988).

In Fig. 9 the hydrophobicity profiles across the DMPC bilayer membrane obtained at –165°C for samples prepared without and with the addition of 10 mol% carotenoids are shown. Instead of lutein we present the profile for another macular xanthophyll, zeaxanthin. For lutein we have only one point at the membrane center (C16 position) and for β-cryptoxanthin we have only two points (C5 and C16 positions) in the profile, that are included in the figure presented here. All hydrophobicity profiles show a typical bell-like shape with a gradual increase of the hydrophobicity towards the bilayer center. The nonpolar β-carotene does not affect the membrane hydrophobicity at any depth, while dipolar carotenoids, zeaxanthin and lutein, increase the hydrophobicity of the membrane center from the level of 1-octanol (ε = 10) to the level of between hexane and dipropylamine (ε = 2–3). We relate the local hydro-

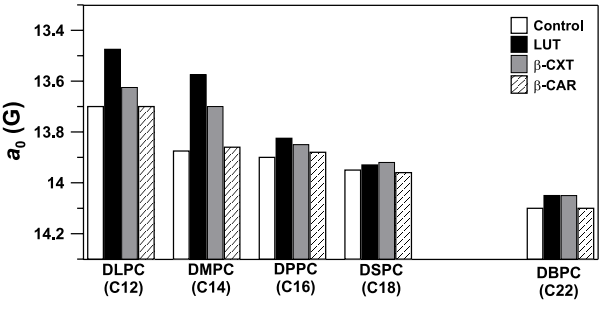

Figure 8. Changes of the isotropic hyperfine coupling constant, a_0, of 16-SASL in PC membranes induced by the addition of 10 mol% carotenoid to the sample.
Results were obtained well above the main phase-transition temperature of PC membranes (see Fig. 6 for temperatures) and plotted as a function of the number of carbon atoms in PC alkyl chains. Taller bars indicate higher hydrophobicity. Because of the sharpness of the EPR lines for 16-SASL and the method of measurements of A'_{II} and $A'_{\perp}$ (see Fig. 3B), the accuracy of measurements of a_0 is ± 0.05 G.

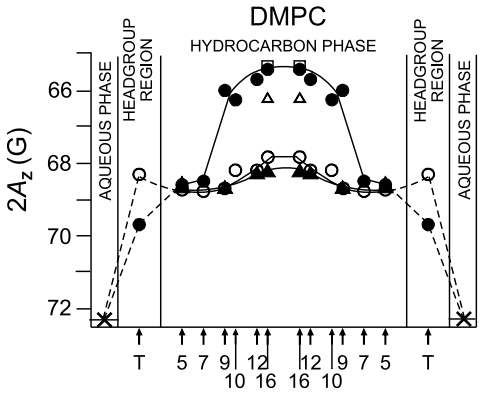

Figure 9. Hydrophobicity profiles ($2A_Z$) across the DMPC membrane.
Profiles were obtained for the membranes without additions (○) and after addition of 10 mol% zeaxanthin (●), lutein (□), β-cryptoxanthin (△), and β-carotene (▲) to the sample. Upward changes indicate increases in hydrophobicity. Note that for β-cryptoxanthin only two measurements were performed, with 5- and 16-SASL, and for lutein only one measurement with 16-SASL was performed. Approximate localizations of the nitroxide moieties of spin labels are indicated by arrows under the base line. For more details see Subczynski *et al*. (1994) and Wisniewska & Subczynski (1998). Data for zeaxanthin and β-carotene were adopted from Wisniewska and Subczynski (1998) and Subczynski and Wisniewska (1998).

phobicity as observed by $2A_Z$ to the hydrophobicity (or ε) of bulk organic solvents by referring to Fig. 2 in Subczynski *et al*. (1994). The effect of β-cryptoxanthin in the membrane center is smaller than that of zeaxanthin. Neither carotenoid affects the hydrophobicity of the hydrocarbon region of the bilayer close to the membrane head group region (C5 position). Our results also indicate that the dipolar carotenoid zeaxanthin decreases the hydrophobicity in the membrane polar head group region as monitored by the increase in the $2A_Z$ value at the Tempo-PC (T-PC) position.

DISCUSSION

Solubility of carotenoids in lipid bilayer membranes

It is commonly assumed that xanthophylls (dipolar, terminally dihydroxylated carotenoids) are well soluble in lipid bilayer membranes. However, in earlier papers the terms: "solubility" and "incorporation" were often used interchangeably. In our discussion we use the term "solubility" to refer to the amount of carotenoids dissolved in the lipid bilayer as monomers, while the term "incorporation" refers to the amount of carotenoids present in the lipid bilayer in the form of monomers, dimers, oligomers and aggregates. To the best of our knowledge, only Socaciu *et al.* (2000; 2002) measured the incorporation ratio of different carotenoids in different membranes finding high incorporation for xanthophylls and low incorporation for β-carotene. Our results indicate that when xanthophylls are added to the samples at an amount lower than 10 mol%, nearly 100% are incorporated. Also, other researchers often assume 100% incorporation of xanthophylls into lipid bilayers (see for example Sujak *et al*., 2000). In more recent works the problems of carotenoid solubility and aggregation within the lipid bilayer were treated more rigorously. The reported threshold of xanthophyll solubility (concentration of xanthophylls at which aggregation initiates) was 10 mol% (Gabrielska & Gruszecki, 1996). However, lower values of xanthophyll solubility, such as 5 and 2 mol%, were reported for small unilamellar vesicles and lipid multibilayers, respectively (Sujak *et al*., 2000; 2002). The earlier reported values of xanthophyll incorporation into fluid-phase model membranes are also near 10 mol% (Milon *et al*., 1986; Lazrak *et al*., 1987), but values as high as 17 mol% (Gruszecki, 1991) and 28 mol% (Kolev & Kafalieva, 1986) have been reported. Recently, we also showed that adding up to 20 mol% lutein to the sample can affect properties of the thick 1-palmitoyl-2-docosahexaenoyl PC membrane without indicating saturation of the observed effect (Wisniewska & Subczynski, 2006a).

It is uncertain how much of β-carotene added to the sample during preparation can be dissolved in the lipid bilayer in the form of monomers. Data exist that at a concentration as low as 0.5 mol%, β-carotene starts to crystallize out in the aqueous buffer (Kennedy & Liebler, 1992), while other researchers report 1 mol% as the final solubility of β-carotene (Woodall *et al*., 1995). These data were confirmed by Socaciu *et al*. (2000), who observed low incorporation of β-carotene into lipid membranes. Our data also indicate low solubility of β-carotene in lipid bilayer membranes (see also Subczynski & Wisniewska, 1998). However, data exist showing that the effect of β-carotene on membrane properties increases with β-carotene content up to 2.5–5.0 mol% (Strzalka & Gruszecki, 1994), up to 10 mol% (Gabrielska & Gruszecki, 1996), and even up to 20 mol% (Jezowska *et al*., 1994). We have no data about the solubility of β-cryptoxanthin in fluid-phase lipid bilayers. However, the obtained data suggest that the solubility of β-cryptoxanthin strongly depends on membrane thickness. The threshold of β-cryptoxanthin solubility in the fluid-phase DLPC membrane can lay around 3 mol%, in DMPC between 5 and 10 mol%, and for thicker membranes can be as large as for lutein.

Because of these solubility problems and uncertainties, we reported the carotenoid concentration as the amount added to the sample during preparation of liposomes and not as the amount dissolved in the lipid bilayer. Carotenoids should affect membrane physical properties mainly when they are dissolved in the lipid bilayer as monomers. Undissolved carotenoid molecules that form aggregates within the lipid bilayer and crystals outside the lipid bilayer, in the water phase, should not affect membrane properties.

We think that the high membrane solubility of macular xanthophylls is one of the major factors that distinguish them from other carotenoids available in the blood plasma. It explains the extremely high concentration of macular xanthophylls in the central fovea, of the order of 1 mM (Landrum *et al*., 1999; Bhosale *et al*., 2004), which is about 1000 times higher than in other tissues. The extremely high level of macular xanthophylls in the eye retina does not reflect their content in the membranes of photoreceptor outer segments. Macular xanthophylls in photoreceptor outer segments constitute about 10% of the amount in the entire retina (Rapp *et al*., 2000), although values as high as 25% have also been reported (Sommerburg *et al*., 1999). In spite of the lower amount, the local concentration of macular xanthophylls in membranes of the rod outer segment is about 70% higher than in residual retinal

membranes (Rapp *et al*., 2000). It is plausible that the macular xanthophylls in Henle's fiber layer mainly filter blue light. One would expect a direct antioxidant function of macular xanthophylls to reside mainly in photoreceptor outer segments. The high solubility of dipolar, terminally dihydroxylated carotenoids in lipid-bilayer membranes could be of great importance for both blue light filtering and antioxidant action.

Orientation of carotenoids in lipid bilayer membranes

A membrane location is one possible site where lutein and zeaxanthin may be accumulated in the eye retina. Based on measurements with model lipid-bilayer membranes, Bone and Landrum (1984) concluded that lutein is located in the membrane and oriented perpendicular to the bilayer surface. This orientation in lipid-bilayer membranes was confirmed for zeaxanthin (Gruszecki & Sielewiesiuk, 1990). However, a later paper from Gruszecki's group (Sujak *et al*., 1999) reported that in model phosphatidylcholine membranes, zeaxanthin and lutein adopt somewhat different orientations in lipid-bilayer membranes, with zeaxanthin situated perpendicular to the membrane surface and lutein existing in two distinct pools in the membrane. One pool adopts the same orientation as zeaxanthin (perpendicular to the membrane surface) and the other adopts a parallel orientation with respect to the membrane surface (for more details see the review by Gruszecki & Strzalka, 2005). Also, different orientations of lutein and zeaxanthin were observed in monolayers formed at the argon-water interface (Sujak & Gruszecki, 2000). However, the orientation of aggregates of lutein and zeaxanthin in lipid bilayer membranes is very similar (Sujak *et al*., 2002). We also investigated the effects of xanthophylls, including lutein and zeaxanthin, on the physical properties of phosphatidylcholine membranes but found no indication of significant differences between these two carotenoids (see Wisniewska & Subczynski, 2006b, and citations therein). On the basis of our own measurements and the fact that the Gruszecki's results were not confirmed by other groups, we assume that lutein and zeaxanthin adopt an orientation mainly perpendicular to the membrane surface (see also the review by Krinsky, 2002). Lutein and zeaxanthin molecules are inclined with respect to the normal to the bilayer surface when the thickness of the bilayer is less than the length of dihydroxycarotenoids, as in DLPC and DMPC membranes (Gruszecki & Sielewiesiuk, 1990). The best match of the length of the dihydroxycarotenoid molecule (the distance between hydroxyl groups is 30.2 Å) with the thickness of the hydrocarbon interior of the PC bilayer is for DPPC and DSPC membranes. This is possibly the reason for the high xanthophyll incorporation into DPPC membranes (28 mol%) reported by Kolev & Kafalieva (1986).

Most uncertain is the orientation of the nonpolar β-carotene in the lipid bilayer. Data presented by van der Ven *et al*. (1984) show that all orientations of β-carotene are possible, parallel to the membrane surface in dioleoyl-PC bilayer, perpendicular to the membrane surface in soy bean-PC bilayer, and with both orientations present in DMPC membranes. Nevertheless, the presence of polar hydroxyl groups ensures the perpendicular orientation of xanthophyll molecules in the bilayer. It is worth noticing that already on the level of lipoproteins in blood plasma the nonpolar β-carotene and lycopene, as well as the monopolar β-cryptoxanthin, are mainly located in the larger, less dense LDL particles, whereas the dipolar lutein and zeaxanthin are located preferentially in the smaller, more dense LDL particles (Lowe *et al*., 1999). Thus already during carotenoid transportation in blood plasma, dipolar carotenoids are segregated from monopolar and nonpolar ones.

The presence of polar hydroxyl groups at the ends of a carotenoid molecule seems to affect the antioxidant properties of carotenoids in the membrane (Woodall *et al*., 1997). Although dipolar zeaxanthin and nonpolar β-carotene show similar antioxidant properties in organic solutions, they differ when incorporated into membranes. Zeaxanthin was shown to react with free radicals slightly more effectively than β-cryptoxanthin and much more effectively than β-carotene (Woodall *et al*., 1997). We think that the perpendicular orientation of dipolar carotenoids in the membrane due to the presence of polar hydroxyl groups at both ends of their molecules may enhance the antioxidant properties of those carotenoids also indirectly, *via* their strong effects on membrane properties. In the presented work, as well as in our previous studies, we have shown that the perpendicularly oriented dipolar carotenoids, but not nonpolar carotenoids (Subczynski & Wisniewska, 1998), affect significantly the membrane physical properties, including membrane fluidity and order of alkyl chains (Subczynski *et al*., 1992; 1993), rotational fluctuations of alkyl chains (Yin & Subczynski, 1996), hydrophobicity of the membrane interior (Wisniewska & Subczynski, 1998) and oxygen transport rate within the membrane (Subczynski *et al*., 1991; Wisniewska & Subczynski, 2006a). These changes in the membrane properties caused by xanthophylls should also be considered as protective against lipid peroxidation, which was discussed in the cited papers.

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